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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A01N 63/00, C07C 315/00 — A1

(11) International Publication Number: WO 99/16318

(43) International Publication Date: 8 April 1999 (08.04.99)

(21) International Application Number: PCT/US98/19972

(22) International Filing Date: 24 September 1998 (24.09.98)

(30) Priority Data:
60/060,235 26 September 1997 (26.09.97) US

(71) Applicant: UAB RESEARCH FOUNDATION [US/US]; Suite 1120G, 701 S. 20th Street, Birmingham, AL 35294-0111 (US).

(72) Inventors: HUANG, Shu, T.; 2607 Foothills Drive, Birmingham, AL 35226 (US). HORTIN, Glen, L.; 14309 Cartwright Way, North Potomac, MD 20878 (US).

(74) Agent: ADLER, Benjamin, A.; McGregor & Adler, 8011 Candle Lane, Houston, TX 77071 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: REDUCED ANTIGENIC CELLS AND USES THEREFOR

(57) Abstract

The present invention provides a composition of matter, comprising a red blood cell coated with a polymeric mixture of polyethylene glycol and albumin. Also provided is a method of blocking blood group antigens on the surface of a red blood cell, comprising the step of contacting said cell with a pharmacologically effective concentration of the composition of the present invention.

09/872,466 CERUS-5100
Adonis Stassinopoulos
Contact: Linda Aston
(925) 288-6107

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REDUCED ANTIGENIC CELLS AND USES THEREFOR

BACKGROUND OF THE INVENTION

Cross-reference to Related Application

This non-provisional patent application claims benefit of provisional patent application U.S. Serial number 60/060,235, filed September 26, 1997, now abandoned.

Field of the Invention

The present invention relates generally to the fields of molecular biology and erythrocyte biochemistry. More specifically, the present invention relates to reduced antigenic cells and uses therefor.

Description of the Related Art

Many polymorphic blood group antigens are expressed on the surface of human red blood cells (Issitt 1985; Mollison, Engelfriet, and Contreras 1987). These include the well-known ABO type and Rh antigen (also termed D antigen) as well as many

other less familiar antigens. As a consequence of the antigenic diversity of red cells, it is not possible to transfuse blood that is antigenically identical with a recipient, unless blood is provided by autologous donation or by an identical twin. Recipients of multiple transfusions develop antibodies against the nonself antigens on red cells.

Alloimmunization also can occur during pregnancy if there is fetal-maternal exchange of blood. As antibodies to other individuals' red cell antigens develop, it becomes progressively harder to provide compatible blood and to avoid transfusion reactions. Transfusion of compatible blood also becomes impossible in patients with autoimmune disorders who produce antibodies against antigens of their own red blood cells. The red cells in individuals with autoimmune disorders and any transfused red cells are destroyed, producing an autoimmune hemolytic anemia which can be fatal in severe cases.

The prior art is deficient in the lack of effective means of producing red cells that are compatible with all recipients and reduced antigenic cells and uses therefor. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

Cell antigens, such as red cell antigens can be masked with a nonantigenic polymer, polyethylene glycol (PEG), as a means of producing red cells are compatible with all recipients, including those with multiple alloantibodies or autoantibodies to red cells. In previous studies, the technique of covalent coupling polyethylene glycol (PEG) derivatives to the surface of red cells as

a means of covering blood group antigens and producing cells that could serve as universal donor cells for transfusion was described. Effective blockade of red cell antigens was achieved with activated esters of polyethylene glycol.

The present invention involves a second generation technique which is an improved procedure in which multiple layers of polyethylene glycol-albumin copolymers are generated instead of single layer of individual polyethylene glycol polymer on red cell surface. This new technique showed less red cell damage and was much more efficient in blocking red cell antigen-antibody reactions. The mouse red cell survival studies showed normal 27 days red cell life span similar to untreated mouse red cells. This new technique offer potential for universal donor red cells for blood transfusion.

In one embodiment of the present invention, there is provided a composition of matter, comprising a red blood cell coated a polymeric mixture of polyethylene glycol and albumin.

In yet another embodiment of the present invention, there is provided a method of blocking blood group antigens on the surface of a red blood cell, comprising the step of contacting said cell with a pharmacologically effective concentration of the composition of the present invention.

In yet another embodiment of the present invention, there is provided a composition of matter, comprising a cell coated with a cross-linked or branched polyethylene glycol and a protein.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not considered limiting in their scope.

Figure 1 shows the polyethylene glycol-albumin polymers treated mice red blood cells have the same survival over 27 days.

Figure 2 shows the polyethylene glycol treated mouse red blood cells only had a 3 day life span. In contrast, untreated red blood cells had a 27 day life span.

Figure 3 shows the red cell survival over 27 days in mice red blood cells treated with the polyethylene glycol-albumin polymers after the red cells were treated in AS-5 additive solution for 7 days. Figure 3 shows the 40% of control untreated mice red blood cells and 60% of the red cell was rapidly cleared within one day. These represent a fraction of damaged red cells after one week storage in AS-5 additive solution that was rapidly cleared. AS-5 additive solution is designed to preserve human red blood cells and may not be optimal for mice red blood cells. The remaining red blood cells after one day transfusion showed similar survival curve when compared to untreated red blood cells.

Figure 4 shows the data shown in Figure 3 except survival is shown as a percentage of cells present at one day transfusion (1d=100%).

Figure 5 shows the red cell morphology after polyethylene glycol-albumin copolymers treatment of human red blood cells in a wet preparation in normal saline (x700). The bottom panel shows control untreated human red blood cells

DETAILED DESCRIPTION OF THE INVENTION

In the present invention, coupling of an inert polymer to the surface of red cells was examined as a means of covering blood group antigens and producing cells that could serve as universal donor cells for transfusion. Effective blockade of red blood cell antigens was achieved with N-hydroxysuccinimide-activated esters of polyethylene glycol. It was possible to block all antigens tested, but lower concentrations of reactants were required to block peptide-defined antigens than carbohydrate-defined antigens. Red cells remained intact after modification but were significantly damaged. The present invention demonstrates the feasibility of antigenic blockade of red cells and that damage can be reduced during coupling reactions to produce viable red cells.

The present invention is directed to a composition of matter, comprising a red blood cell coated with an inert, nonantigenic polymer. Preferably, the polymer is a N-hydroxysuccinimide-activated esters of polyethylene glycol. The polymer may be a bis(propionyl-N-hydroxysuccinimide)-polyethylene glycol. Alternatively, the polymer is propionyl-N-

hydroxysuccinimide-methoxypolyethylene glycol with a molecular weight of 5,000. Preferably, the red blood cell coated with an inert, nonantigenic polymer has been treated with a concentration of from about 1% of the polymer.

The present invention is also directed to a method of blocking blood group antigens on the surface of a red blood cell, comprising the step of contacting said cell with a pharmacologically effective concentration of the composition of the present invention. Preferably, the blocking of the blood group antigens on the surface of the cell significantly reduces the antigenicity of such red blood cells when administered to an individual in need of such treatment. Preferably, the polymer is a N-hydroxysuccinimide-activated esters of polyethylene glycol.

The polymer may be a bis(propionyl-N-hydroxysuccinimide)-polyethylene glycol. Alternatively, the polymer is propionyl-N-hydroxysuccinimide-methoxypolyethylene glycol with a molecular weight of 5,000. Preferably, the red blood cell coated with an inert, nonantigenic polymer has been treated with a concentration of about 1% of the polymer.

Many polymorphic blood group antigens are expressed on the surface of human red blood cells (Issitt 1985; Mollison, Engelfriet, and Contreras 1987). These include the well-known ABO type and Rh antigen (also termed D antigen) as well as many other less familiar antigens. As a consequence of the antigenic diversity of red cells, it is not possible to transfuse blood that is antigenically identical with a recipient, unless blood is provided by autologous donation or by an identical twin. Recipients of multiple transfusions develop antibodies against the nonself antigens on red cells. Alloimmunization also can occur during

pregnancy if there is fetal-maternal exchange of blood. As antibodies to other individuals' red cell antigens develop it becomes progressively harder to provide compatible blood and to avoid transfusion reactions. Transfusion of compatible blood also becomes impossible in patients with autoimmune disorders who produce antibodies against antigens of their own red blood cells. Their own red cells and any transfused red cells are destroyed, producing an autoimmune hemolytic anemia which can be fatal in severe cases. It is also important to note that not all antigens need to be blocked on each cell for the present compositions to have utility. For example, one may only block Rh determinant on cells which could be useful for autoimmune hemolytic problems.

The masking of red cell antigens with a nonantigenic polymer, polyethylene glycol (PEG), as a means of producing red cells compatible with all recipients, including those with multiple alloantibodies or autoantibodies to red cells is shown herein. The first generation technique which conjugated polyethylene glycol polymer to the red cell surface to decrease red cell agglutination showed only partial blockage of red cell antigen-antibody reaction and moderate damage of red cell membrane. Significant reduction in red cell damage resulted from use of a group of polyethylene glycol reagents that were of higher molecular weight. However, blockade of antigens were still incomplete.

The present invention shows the development of a second generation improved technique which conjugates a small amount of polyethylene glycol-albumin copolymers on the red cell surface first with subsequent deposition of layer by layer polyethylene glycol-albumin copolymers on top of the existing polymers on red cell surface. This new technique showed that all

of the red cell antibodies could be completely blocked, except ABO antibodies which showed very weak microscopic reactions. Developing layers of cross-linked PEG-albumin copolymers to cover the red cell surface is much thicker and much more efficient in covering the red cell antigens then polyethylene glycol polymer alone. Also, these new polyethylene glycol-albumin copolymers have less attached points on red cell surface, therefore causing less damage to the red cells. This technique can be used to create universal donor red cells for blood transfusion, since it showed efficient blockage of red cell antigen-antibody reaction while preserving normal red cell morphology and red cell survival.

Thus, the present invention relates to a composition of matter, comprising a red blood cell coated a polymeric mixture of polyethylene glycol and albumin. Generally, any protein or compound containing multiple reaction sites with activated esters which form a cross-linked matrix can be used to create the universal donor blood cells of the present invention. Preferably, the concentration of the polyethylene glycol is from about 0.5% to about 1.0%. In addition, the preferable concentration of the albumin is from about 1.25% to about 2.5%. Representative examples of suitable albumin formulations include Albumarc™ 5% from the American Red Cross Blood Services and Albutein® 5% from the Alpha Therapeutic Corporation. Preferably, the cell is coated with the polymeric mixture during one single reaction although multiple treatments could be applied.

The present invention relates to a method of blocking blood group antigens on the surface of a red blood cell, comprising the step of contacting said cell with a pharmacologically effective concentration of the composition disclosed herein. Preferably, the

concentration of the polyethylene glycol is from about 0.5% to about 1.0%. In addition, the preferable concentration of the albumin is from about 1.25% to about 2.5%. Representative examples of suitable albumin formulations include Albumarc™ 5% from the American Red Cross Blood Services and Albutein® 5% from the Alpha Therapeutic Corporation. Preferably, the cell is coated with a polymeric mixture one time so as to allow the interactive polyethylene glycol and albumin copolymer to develop thicker and thicker polymers on the red cell surface. This procedure can be repeated if necessary.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Materials and methods

Polyethylene glycol (PEG) derivatives were purchased from Shearwater Polymers (Huntsville, AL). Red blood cells were obtained from segments attached to units of donor red blood cells. Imucor (Norcross, GA) was the source of monoclonal/polyclonal anti-human globulin, antibodies to human red cell antigens. Fresh porcine blood was obtained from the Animal Care Facility. Lectins were purchased from Gamma Biologicals (Houston, TX). Red blood cell bound antibody was measured by the UAB Immunecytopenia Laboratory using an immunoassay as previously described by LoBuglio et al (1983). Agglutination reactions of red cells were assayed by standard tube techniques as described by the Technical Manual for blood banks (Walker et al., 1990).

Quantitative estimates of the strength of agglutination reactions after serial dilution of the agglutinating serum. Strength of agglutination is expressed as a titer scores which relates to the inverse of the dilution yielding agglutination.

Coupling of activated polyethylene glycols to red blood cells was conducted in 140 mM NaCl, 20 mM N-[2-hydroxyethyl)piperazine-N'[2-ethanesulfonic acid] (HEPES) adjusted to pH 8.0. A 5% suspension of red blood cells was mixed at 4°C with activated polyethylene glycol derivatives added to varying percentages by weight. Incubations were at 4°C overnight. Cells were washed in a centrifugal cell washer before any analysis.

EXAMPLE 2

Results

Incubation of red cells overnight with the bifunctional activated polyethylene glycol derivative, bis(propionyl-N-hydroxysuccinimide)-polyethylene glycol of 3,400 daltons progressively blocked agglutination reactions by antibodies to blood group antigens such as A, A'B, and D antigens as the concentration of reagent was increased (TABLE I). The D antigen, which is one of the polypeptide-defined determinants of the Rh system, was blocked at a lower concentration than required for antigenic determinants defined by carbohydrate groups, e.g., the very high concentration of reagent required to block the A and A'B antigens. It was difficult to achieve 100% blockade of agglutination against these high abundance, carbohydrate-defined blood group antigens. Preferential blockade of polypeptide-defined antigens is not surprising considering the preferential

reactivity of the active carboxy esters with amino rather than hydroxyl groups (Zalipsky and Lee 1992). Polypeptides may be preferred sites of covalent coupling of the activated polyethylene glycols on the surface of red cells.

TABLE I

Blockade of blood group antigens by coupling with bis(propionyl-N- hydroxysuccinimide)-polyethylene glycol of 3,400 daltons

Titer Scores for Agglutination of Red Cells

%PEG	And-A	Anti A'B	Anti-D
0	2048	1024	256
10	2048	1024	0
20	1024	512	0
30	32	64	0
40	16	16	0
50	2	4	0

Agglutination reactions of polyethylene glycol-modified red cells bearing the A, A'B, and D antigens with serial dilutions of antisera versus the respective antigens were examined.

EXAMPLE 3

It was expected that use of a reagent with a large molecular size would prevent entry of the reagent into cells and would minimize modification or damage to internal components of cells. There was a limited amount of hemolysis of cells, and there was little change in the size distribution of red cells when analyzed with an electronic hematology analyzer. However, cells did undergo some damage as evident by more irregular shapes

microscopically (not shown). Also, breakdown of the red cell membrane as a permeability barrier was suggested by lack of lysis of modified red cells by hypotonic solutions.

Agglutination reactions are an imperfect means of assessing antigenic blockade as agglutination reactions may be influenced by factors such as changes in surface charge of red cells or shape of cells. For this reason, an independent means of measuring the blockade of a red cell antigen was employed. Red cells expressing the D antigen were modified by coupling with the bifunctional polyethylene glycol derivative. Modified cells and control cells were then incubated with antibody to the D antigen, and the amount of cell-bound antibody was measured by immunoassay. This analysis shown in TABLE II indicated a complete blockade (more than 99%) of antibody binding to the D antigen.

TABLE II

Blockade of D antigen by coupling of bis(propionyl-N-hydroxysuccinimide)-polyethylene glycol to red cells.

Red Cell Type	Molecules IgG/Cell
A, Rh Negative (negative control)	396
A, Rh Positive (positive control)	11,233
A, Rh Positive, polyethylene glycol-modified	407

Cells were incubated with antibody to D antigen and antibody molecules bound measured by immunoassay. Rh negative cells lack D antigen and serve as a negative control.

EXAMPLE 4

Blockade of a variety of other antigens by attachment of polyethylene glycol to red cells was examined (TABLE III). Agglutination by antibodies to antigens of the Duffy (Fy), Kidd (Jk), Lewis (Le), and Rh groups (D) were inhibited as well as agglutination reactions by the lectins from *Dolichos bylorous* and *UIex europaens.* These results indicate a generalized blockade of red cell antigens. The antigens most resistant to blockade by red cell modifications with polyethylene glycol have been the A and B antigens, which probably reflects their high abundance, peripheral location, and carbohydrate nature (Walker, 1990).

TABLE III

Blockade of red cell antigens by coupling of bis(propionyl-N-hydroxysuccinimide)-polyethylene glycol

	Agglutination Reaction	
Anfibody/Lectin	Untreated Cells	PEG-Red cells
Type A_1, Rh+ cells treated with 5 % PEG derivative		
Anti-D	4+	0
Anti-Fy^b	1+	0
Anti-Jk^a	2+	weak +
Anti-Le^b	2+	0
Dolichos biflorous	4+	0
UIex europaeus	4+	0
Type A_1B cells treated with 25 % PEG derivative		
Anti-B	4+	1+
Anti~Jkb	1+	0
Anti~Leb	3+	0

EXAMPLE 5

Human serum has naturally-occurring antibodies to red cells of many species, such that xenotransfusion of red cells results in rapid agglutination or lysis of the cells by complement fixation. Blockade of porcine red cells by coupling of a polyethylene glycol derivative was examined as compatibility with human serum. Both agglutination and hemolytic actions of human serum were blocked by the modification of porcine red cells with polyethylene glycol.

TABLE IV

Blockade of porcine red cell antigens by propionyl-N-hydroxysuccinimide-methoxypolyethylene glycol (MW: 5,000)

%PEG	Agglutination	Hemolysis
0	4+	Moderate
5%	3+	Slight
10%	0	None
20%	0	None
30%	0	None
40%	0	None
50%	0	None

Modified porcine red cells were mixed with human serum and agglutination and lysis reactions were assessed.

EXAMPLE 6

Polyethylene glycol derivatives were purchased from Shearwater Polymers (Huntsville, AL). The 5% human albumin

was purchased from American Red Cross Blood Services. Red cells were obtained from segments attached to units of donor red cells. Immucor (Norcross, GA) was the source of monoclonal/polyclonal anti-human globulin, antibodies to human red cell antigens. Agglutination reactions of red cells were assayed by standard tube techniques as described by the Technical Manual of for Blood Banks (Walker et al 1994).

Coupling of activated polyethylene glycols to red cells was conducted in 140 mM NaCl, 20 mM N-[2-hydroxyethyl)piperazine-N'[2-ethanesulfonic acid] (Hepes solution) adjusted to pH 9.0. Activated refers to polyethylene glycol which has chemically reactive esters attached. A 5% human albumin was mixed at room temperature with activated branched polyethylene glycol derivatives such as 4 arm branched succinimidyl derivative of PEG propionic acid (SPA-PEG) or specific product thereof- 4 arm propionyl-N-hydroxysuccinimide-methoxypolyethylene glycol MW 10,000) for one minute and then mixed with 5% suspension of red cells for 20 minutes and then fresh Hepes solution was added for developing polyethylene glycol-albumin copolymer layers for another 25 minutes. Cells were washed with PBS, pH 7.0 in a centrifugal cell washer before any analysis. The red cell survival studies using PKH-2 fluorescent dye technique as described by Read et al. in Transfusion Journal 31(6): 502-508, 1991.

EXAMPLE 7

Hemagglutination tests

TABLE V shows that polyethylene glycol-albumin copolymers completely blocked all red cell antigen-antibody

reactions except very weak microscopic agglutination with anti-A, anti-B and anti-A'B antibodies. Testing with polyclonal allo-antibodies such as anti-K, anti-FyA, anti-FyB, anti-JkA, anti-JkB, anti-S, anti-s and other Rh antibodies, showed same results as above which indicated a complete block of antigen-antibody reaction. Testing with anti-B and anti-A'B showed same result as above, negative on IS and 30'RT but micro + on anti-IgG phase.

TABLE V

Testing with polyclonal anti-D:

	IS	15'37C	anti-IgG
A+ untreated (control)	4+		
A+ PEG-Albumin treated	0	0	0
A- untreated (control)	0	0	0
A- PEG-Albumin treated	0	0	0

Testing with human polyclonal anti-A:

			30'37°C	
	IS	30'RT	anti-IgG	Titer at RT
A- untreated (control)	4+	4+	4+	128
A-PEG-Album. treated	0	0	micro +	

Testing with monoclonal anti-A:

	IS	30'RT
A- untreated (control)	4+	4+
A- PEG-Albumin treated	0	micro +

EXAMPLE 7

Red cell survival studies

The red cell survival studies showed polyethylene glycol-albumin treated mouse red blood cells have the same 27 days life span compared with untreated red blood cells (Figure 1). In contrast, polyethylene glycol treated mouse red blood cells only have 3 days life span (Figure 2). Polyethylene glycol-albumin treated mouse red blood cells (in AS-5 additive solution, a solution containing dextrose, mannitol, adenine and sodium chloride that is use to preserve human red blood cells so that such cells can be stored for long periods of time) was stored in 4°C refrigerator for one week. When transfused back into mouse, there was a loss of 60% of transfused red blood cells in first day and the remaining red cells had a 24 day life span. The untreated control mouse red cells showed loss of 40% of transfused red blood cells in first day and the remaining red cells have 27 days life span (Figures 3 and 4). The AS-5 additive was not the optimal additive solution for mouse red cells, as it showed immediate large red cell destruction on both treated and untreated mouse red cells and a slight decrease of remaining red cell survival on polyethylene glycol-albumin treated compare with untreated mouse red cells.

EXAMPLE 8

Morphology of red blood cells

Figure 5 shows that both polyethylene glycol-albumin treated and untreated control human red blood cells showed normal red cell morphology in microscopic examination. The present invention showed that polyethylene glycol-albumin polymers are more efficient than polyethylene glycol polymer in

covering the antigen sites on red cell surface to prevent antibodies reaction. Polyethylene glycol polymer is a single layer coated on the red cell surface, with space between each polymer which allow allows the antibody to reach the antigens. In contrast, the polyethylene glycol-albumin copolymers are multiple layers and have sheet-like structures due to cross-linked or branched polyethylene glycol and albumin polymers. These sheet-like structures prevent the antibody from reaching the antigens with the exception of ABO antibodies as ABO antigens are far extended out from the surface. The polyethylene glycol-albumin copolymer had more efficient coverage on red cell surface and have less attached points on red cell surface which showed normal red cell morphology and red cell survival studies indicating very minimal damage to the red cell surface. The polyethylene glycol-albumin copolymers exhibit high potential as universal donor red cells for blood transfusion.

Thus, the present invention provides a composition of matter, comprising a red blood cell coated with a cross-linked or branched polyethylene glycol and albumin. Generally, any branched polyethylene glycol should be useful in creating the present compositions. Preferably, "arm" polyethylene glycols which has multiple molecules connnected together with multiple actives sites are preferred. For example, 4 arm refers to 1 PEG of a MW of about 3400-5000; 4 arm refers to 4 PEG molecules of about 15,000-20000 MW. Four PEGs connected together would have therefore 4 active sites.

Generally, the concentration of the polyethylene glycol is from about 0.5% to about 1% and the concentration of the albumin is from about 1.25% to about 10%. In a preferred form,

the albumin is human albumin. In another aspect, the present invention can provide a white blood cell coated with cross-linked mixture of branched polyethylene glycol and albumin so as to block the antigen sites on such a cell. For all of the compositions described herein, albumin may be preferred but other similar proteins could easily be used, e.g., fibrinogen.

Generally, in making the compositions described herein, the polyethylene glycol is mixed with the albumin to form a copolymer. Subsequently, the cells such as red blood cells are added. Over a short period of time, such as 20 minutes, the copolymer attaches to the red blood cell blocking the antigen sites. The cell is coated with polymeric mixture at least one time.

The present invention also provides a method of blocking blood group antigens on the surface of a red blood cell, comprising the step of contacting said cell with a pharmacologically effective concentration of the composition described herein. The present invention is also directed to a composition of matter, comprising a red blood cell coated with a cross-linked or branched polyethylene glycol and branched polyethylene glycol amine. The present invention is also directed to a composition of matter, comprising a red blood cell coated with a cross-linked or branched polyethylene glycol and an albumin-like compound, wherein said compound contains multiple reaction sites which form a cross-linked matrix with activated esters of polyethylene glycol.

The present invention is further directed to a composition of matter, comprising a cell coated with a cross-linked or branched polyethylene glycol and a protein. Preferably, the cell is a red or white blood cell but could be any cell type. In one

embodiment, the protein is albumin but could be any protein contains multiple reaction sites which form a cross-linked matrix with of polyethylene glycol. In a preferred embodiment, the polyethylene glycol is a branched polyethylene glycol.

EXAMPLE 9

Different kinds of branched amino-PEG (4 arm branched amino-PEG m.w. 10,000 and 2 arm branched amino-PEG m.w. 3,400 obtained from Shearwater) were also used to replace albumin. This data supports that any protein or compound containing multiple reaction sites with activated esters which form a cross-linked matrix – equivalent substitutes for albumin can be used to create the universal donor blood cells of the present invention. Table I showed no differences using albumin or amino-PEG to conjugate with PEG to red blood cells to block antigen-antibody reactions.

TABLE VI

Testing with Monoclonal anti-D:

	IS	15'37°C	anti-IgG
A+ untreated (control)	4+	4+	4+
A-PEG-Albumin treated	0	0	0
A+ PEG-2 branched amino-PEG treated	0	0	0
A+ PEG-4 branched amino-PEG treated	0	0	0
A- untreated (control)	0	0	0

Testing with polyclonal allo-antibodies (anti-FyA, anti-FyB, anti-JkA, anti-JkB, anti-K, anti-S, anti-s) showed the same results as above, indicating a complete block of antigen-antibody reaction

TABLE VII

Testing with human polyclonal anti-A:

	IS	30'RT	30'37°C anti-IgG	Titer at RT
A-untreated (control)	4+	4+	4+	128
A-PEG-Albumin treated	0	0	0 (micro +)	
A+ PEG-2 branched amino-PEG treated	0	0	0 (micro +)	
A+ PEG-4 branched amino-PEG treated	0	0	0 (micro +)	

Testing with anti-B and anti-A'B showed the same results as above, negative on IS and 30'RT, but microscopic + on anti-IgG phase.

In another study, human lymphocytes were treated with PEG-albumin copolymer as the same method used for red blood cells and then lymphocyte antigens (HLA antigens) were tested using standard microlymphocytotoxicity typing methods on pre- and post-treated lymphocyte suspension. The results are shown in Table VIII.

TABLE VIII

HLA antigen specificity	HLA antibody number in tray	Reading Score*** Untreated	Reading Score*** PEG Treated**	10 D*
A11	1F	8	8	8
A11	2F	8	1	1
A11	2E	6	6	8
A11	2D	4	1	1
A11	2C	4	1	1
A11	5D	4	1	1
A24	4B	8	6	6
A24	5A	8	1	1
A24	5B	8	8	8
B62	1E	8	2	1
B62	1F	8	1	1
B62	2F	4	1	1
B62	2E	6	1	1
B62	2A	8	1	1
B62	7D	8	4	8
B62	7E	8	1	1
BW4	10E	6	1	1
BW6	10D	8	4	1
BW6	10C	8	2	1
BW6	10B	8	1	1
BW6	10A	8	8	8

* Lymphocytes treated with PEG-albumin copolymer and incubated in RPMI for 10 d. ** Lymphocytes (HLA typing: A11, A24, B 62, BW4 & BW6) treated with PEG-albumin copolymer. ***Score 1 and 2 = negative reaction; 4=weak reaction; 6=moderate reaction; 8=strong reaction

The results contained in Tables VI, VII and VIII show that the PEG-albumin copolymer conjugated to lymphocyte surfaces could block or decrease exposure of antigens to most HLA antibodies. This blocking effect can continue at least 10 days. These results support that this method can not only block red cell surface antigens, but also block other cell's surface antigens from reacting to various antibodies.

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Read, E.J., Cardine, L.L., Yu, M.Y. (1991) Flow cytometric detection of human red cells labeled with a fluorescent membrane label: potential application to in vivo survival studies. Transfusion 31(6): 502-508

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A composition of matter, comprising a red blood cell coated with a cross-linked or branched polyethylene glycol and albumin.

2. The composition of claim 1, wherein said branched polyethylene glycol is an arm polyethylene glycol.

3. The composition of claim 2, wherein said arm polyethylene glycol is a 4 arm polyethylene glycol.

4. The composition of claim 3, wherein said arm polyethylene glycol is 4 arm propionyl-N-hydroxysuccinimide-methoxypolyethylene glycol.

5. The composition of claim 1, wherein the concentration of said polyethylene glycol is from about 0.5% to about 1%.

6. The composition of claim 1, wherein the concentration of said albumin is from about 1.25% to about 10%.

7. The composition of claim 1, wherein said albumin is human albumin.

8 The composition of claim 1, wherein the cell is coated with polymeric mixture at least one time.

9. A method of blocking blood group antigens on the surface of a red blood cell, comprising the step of contacting said cell with a pharmacologically effective concentration of the composition of claim 1.

10. The method of claim 9, wherein the concentration of said polyethylene glycol is from about 0.5% to about 1%.

11. The method of claim 9, wherein the concentration of said albumin is from about 1.25% to about 10%.

12. The method of claim 9, wherein said albumin is human albumin.

13. A composition of matter, comprising a red blood cell coated with a cross-linked or branched polyethylene glycol and branched polyethylene glycol amine.

14. A composition of matter, comprising a red blood cell coated with a cross-linked or branched polyethylene glycol and an albumin-like compound, wherein said compound contains multiple reaction sites which form a cross-linked matrix with activated esters of polyethylene glycol.

15. A composition of matter, comprising a cell coated with a cross-linked or branched polyethylene glycol and a protein.

16. The composition of claim 15, wherein said cell is a red blood cell.

17. The composition of claim 15, wherein said protein is albumin.

18. The composition of claim 15, wherein said polyethylene glycol is a branched polyethylene glycol.

19. The composition of claim 15, wherein said protein contains multiple reaction sites which form a cross-linked matrix with of polyethylene glycol.

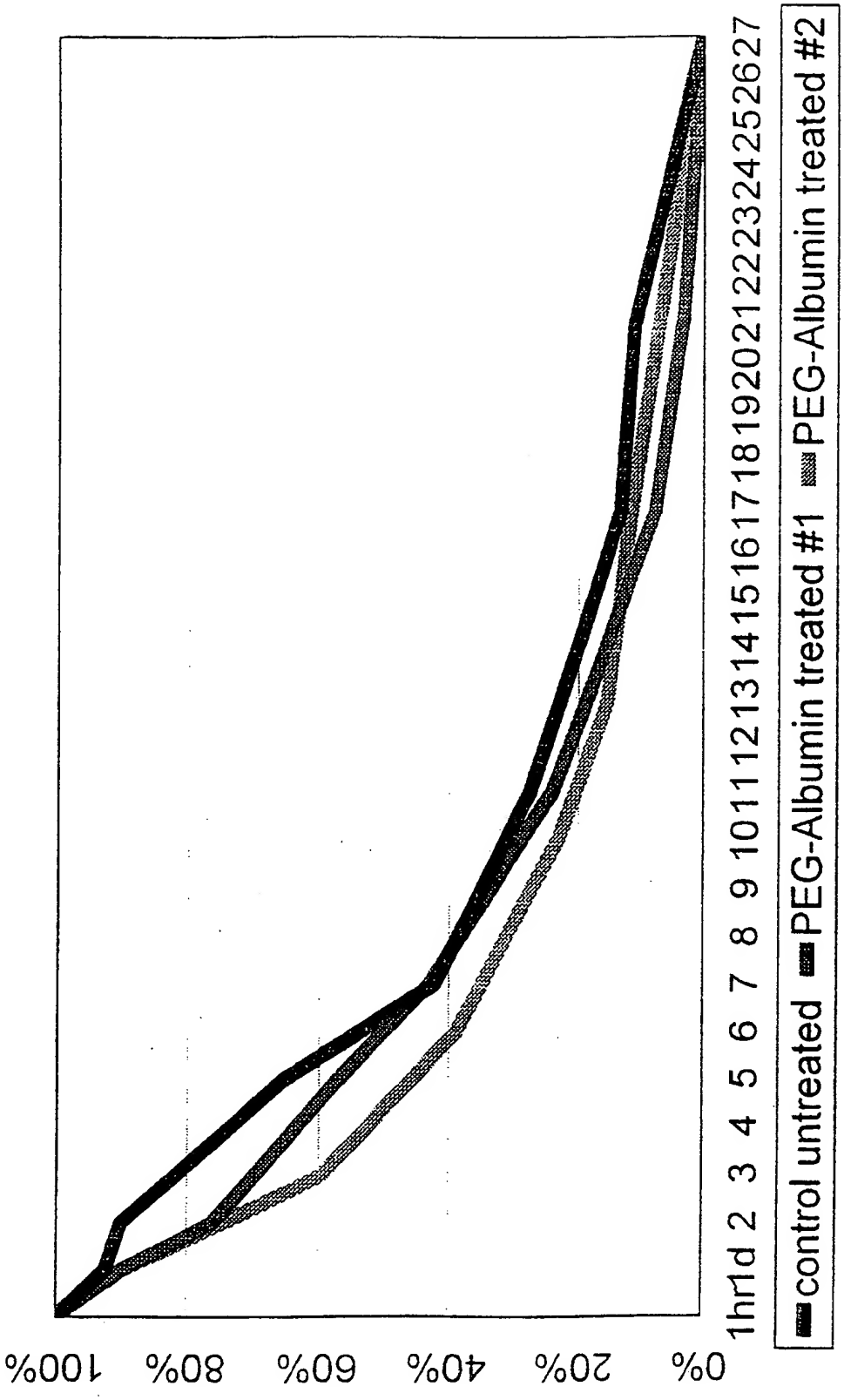

0%
20%
40%
60%
80%
100%
1hr1d 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
control untreated
PEG-Albumin treated #1
PEG-Albumin treated #2

FIGURE 1

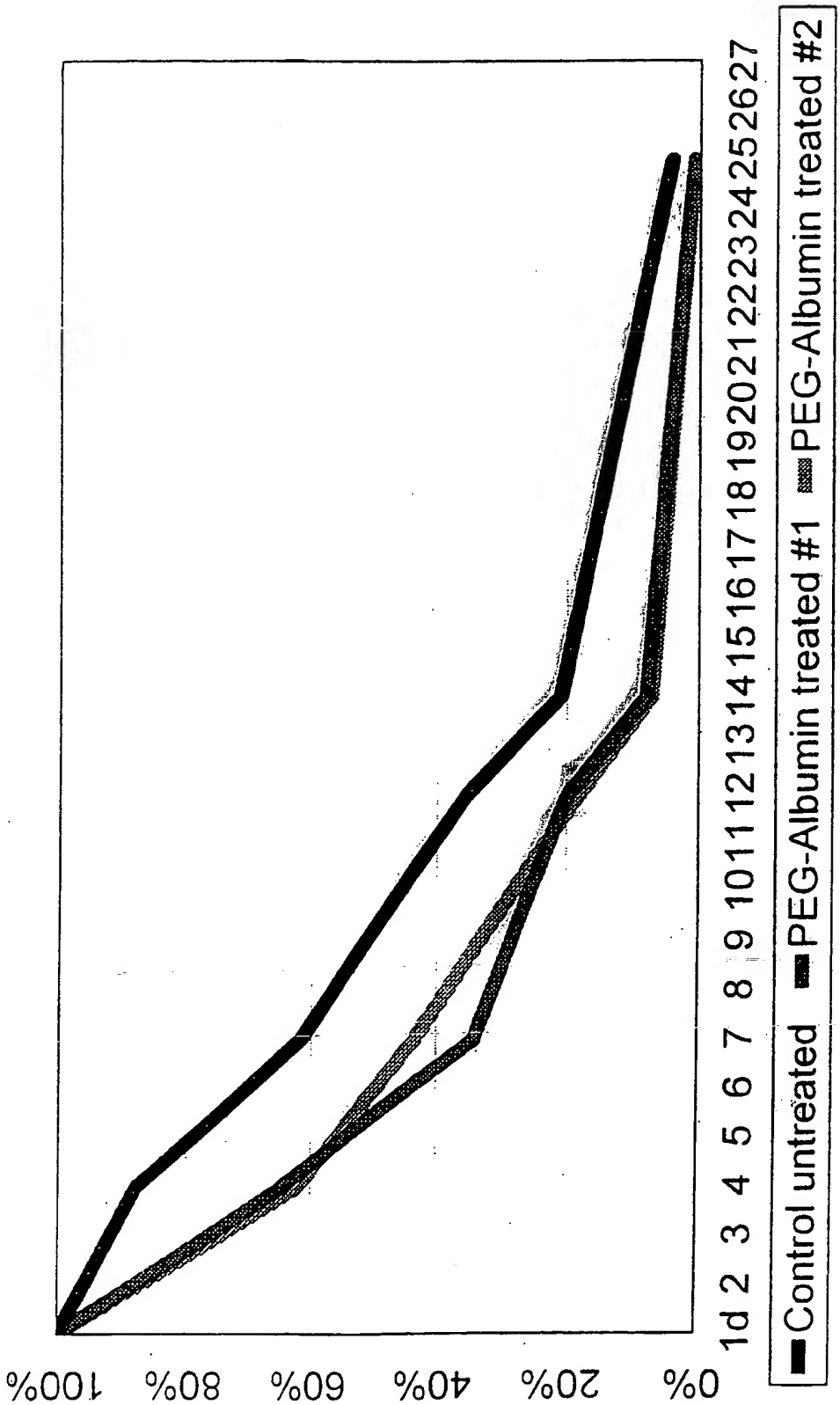
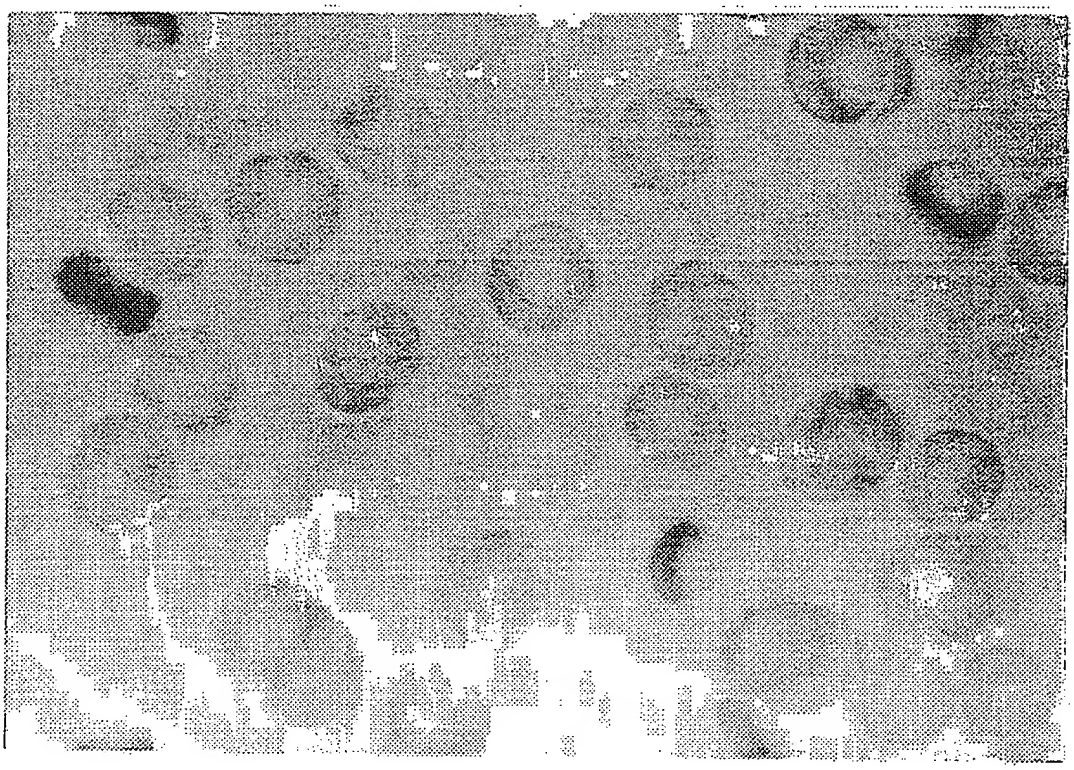

0%
20%
40%
60%
80%
100%
1d 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Control untreated
PEG-Albumin treated #1
PEG-Albumin treated #2

FIGURE 4



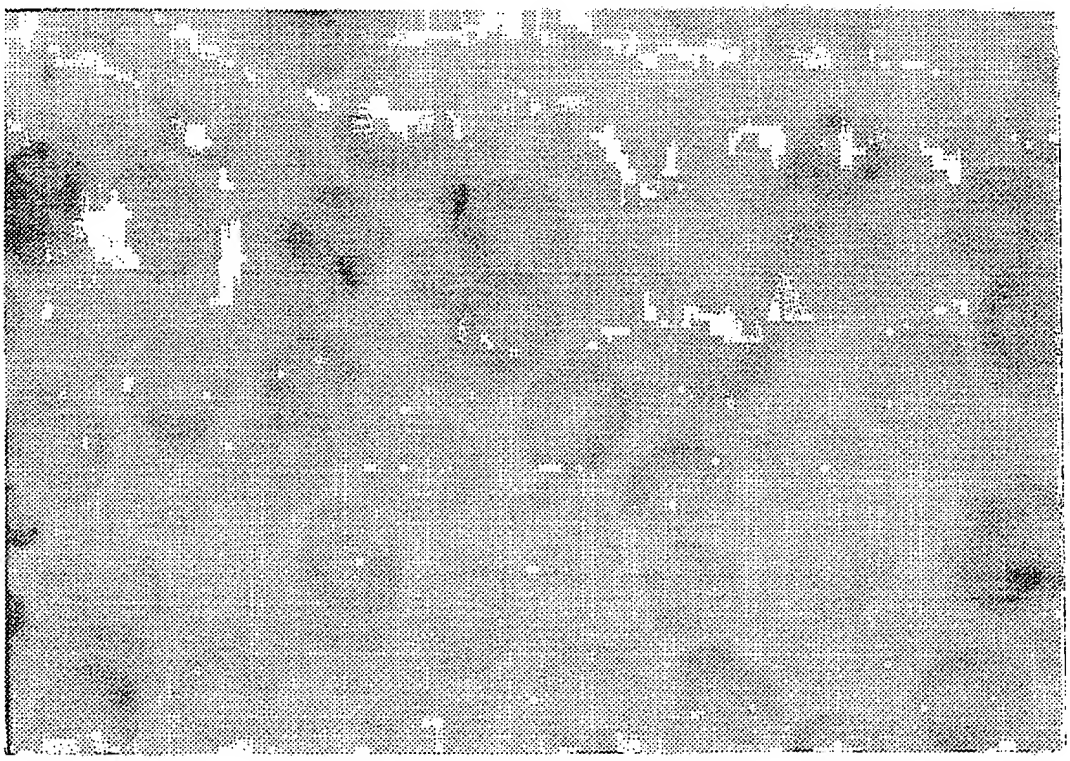


FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No. PCT/US98/19972

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 63/00; C07C 315/00

US CL : 424/93.73;568/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.73;568/32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CAPLUS, EMBASE, BIOSIS search terms: polyethylene glycol, albumin, erythrocytes, red blood cells

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HORTIN et al. Surface-Pegylated red cells as potential universal donor red cells. Blood. 15 November 1996, Vol. 88, No. 10, Suppl. 1 (Part 1 of 2), Abstract 156-II, page 181a.	1-12, 14-19
Y	MURAD, K. et al. Molecular camouflage of antigenic determinants on intact mammalian cells: possible applications to transfusion medicine. Blood. 15 November 1996 Vol. 88, No. 10, Suppl. 1 (Part 1 of 2), Abstract 1765 page 444a.	1-12, 14-19
Y	US 5,498,421 A (GRINSTAFF et al) 12 March 1996, see entire document especially column 3, lines 45-54, column 8, line 56 to column 9, line 2, column 12 line 61 to column 13, line 25 and column 16, lines 33-40, Examples 8, 10, 15, 19 and 20, Claims 1, 2, 15, 17-18 and 22.	1-12, 14-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
24 NOVEMBER 1998	29 DEC 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RONALD P PELLEY Telephone No. (703) 308-9343

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No. PCT/US98/19972

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCOTT et al. Chemical camouflage of antigenic determinants: Stealth erythrocytes. Proc. Natl. Acad. Sci. USA. July 1997, Vol. 94, pages 7566-7571.	1-12, 14-19
Y	US 5,171,264 A (MERRILL) 15 December 1992, see entire document especially column 1, line 65 to column 2, line 23, column 3, line 61 to column 4, line 6, column 4 lines 54 to 65, column 6, lines 18 to 27, and column 8, lines 32 to 36.	1-12, 14-19
T,E	Shearwater Polymers, Inc. Internet advertisement, www.swpolymers.com, 25 November, 1998. Introduction to Shearwater Polymers, Brief review of PEG applications, Star PEG's and Branched PEG's.	1-12, 14-19
Y	US 4,766,106 A (KATRE et al.) 23 August 1988, see entire document especially column 1, lines 36-39, column 1, lines 42-45, column 1, lines 53-68, column 3, lines 36-44, column 4, lines 37-41, column 9, lines 53-63, Example I, A and F, Example VIIIA, and claims 1, 4, 5.	4
A	GRAHAM, N.B. 'Poly (Ethylene Glycol) Gels and Drug Delivery.' In: Poly(Ethylene Glycol) Chemistry Biotechnical and Biomedical Applications. Edited by J. Milton Harris. New York: Plenum Press. 1992, pages 263-281.	1-4, 8, 14-15, 18-19
A	HARRIS et al. 'Synthesis of New Poly (Ethylene Glycol) Derivatives' In: Poly(Ethylene Glycol) Chemistry Biotechnical and Biomedical Applications. Edited by J. Milton Harris. New York: Plenum Press. 1992, pages 371-381.	4
A	SEHON, A.H. 'Suppression of Antibody Responses by Conjugates of Antigens and Monomethoxypoly(Ethylene Glycol).' In: Poly(Ethylene Glycol) Chemistry Biotechnical and Biomedical Applications. Edited by J. Milton Harris. New York: Plenum Press. 1992, pages 139-151.	1-12, 14-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19972

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-12 and 14-19

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19972

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s)1-12 and 14-19, drawn to a composition of matter comprising a red blood cell coated with a cross-linked or branched polyethylene glycol and albumin and a method of blocking blood group antigens using polyethylene glycol and labumin.

Group II, claim(s) 13, drawn to a composition of matter comprising a red blood cell coated with a cross-linked or branched polyethylene glycol and branched polyethylene glycol amine.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Inventions I and II are different because the special technical feature for group I (albumin as crosslinker or branched or star PEG's) differs in its physical and chemical properties from the special technical feature for group II (a polyethylene glycol amine as crosslinker for branched or star PEG's).

Since the special technical feature of the Group I invention - a protein crosslinker - is not present in the Group II invention being claimed and the special technical feature of the Group II invention - a polyoxyethylene amine crosslinker - is not present in the Group I invention being claimed, unity of invention is lacking.